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GRANT NO: DAMD17-94-J-4441

TITLE: Initiation of Breast Cancer by Endogenous Estrogens: Mechanism and Prevention

PRINCIPLE INVESTIGATOR: Jack Fishman, Ph.D.

CONTRACTING ORGANIZATION: Cornell University Medical College
New York, NY 10021

REPORT DATE: July 30, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Material Command
Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	30 Jul 95	Annual 01 Aug 94 - 31 Jul 95

4. TITLE AND SUBTITLE
Initiation of Breast Cancer by Endogenous Estrogens: Mechanism and Prevention

5. FUNDING NUMBERS
DAMD 17-94-J-4441

6. AUTHOR(S)
J. Fishman, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cornell University Medical College
New York, New York 10021

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and Material Command
Fort Detrick, Maryland 21701-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Prepared in cooperation with Dr. C.P. Martucci and Dr. J. Leonard

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

12. ABSTRACT (Maximum 200 words)

We have carried out experiments in cell culture systems demonstrating the unique ability of 16α-OHE_1 to bring about hyperproliferation with increased anchorage-independent growth. This, as well as other studies, has indicated the carcinogen like activity of 16α-OHE_1. We have also carried out studies with mouse and human explant cultures of two types (high and low risk for breast cancer) and a marked increase in 16α-hydroxylation was observed with cultures from the high risk mice or women. The differences demonstrated in these normal tissues indicate the importance of a chronic increase in estrogen 16α-hydroxylation prior to the development of breast tumors.

Experiments with 16α-OHE_1 in the control of PS2 gene expression in MCF-7 cells demonstrate that an "irreversible" interaction between estrogen receptors and 16α-OHE_1 can cause sustained response in estrogen-responsive genes. Also, experiments to identify the cytochrome P450 gene which catalyzes estrogen 16α-hydroxylation have indicated that it is probably Cyp2-dx. Further studies should confirm whether or not it is this gene which is responsible for the enzyme. Once identified it may be possible to alter the expression of this gene in breast cells and therefore reduce the risk of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Estrogen metabolites, covalent binding, estrogen receptor, enzyme manipulation, breast cancer	39
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	unlimited

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8/22/95

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TABLE OF CONTENTS

Title: Initiation of Breast Cancer by Endogenous Estrogens: Mechanism and Prevention

P.I.: Jack Fishman, Ph.D.

I. Introduction

A. Nature of the Problem

While there is general consensus for a role for estrogens in breast cancer, the biological mechanism for it is unknown. This proposal presents a novel biological rationale for the estrogen breast cancer link. We have obtained considerable evidence derived from in vivo and in vitro studies that 16α-hydroxyestrone (16α-OHE1), a metabolite of estradiol, which uniquely among all endogenous estrogens can participate in a specific covalent interaction with the estrogen receptor (1) and is involved in estrogen target cell transformation (2). Based on this concept a strategy which diminishes the formation of this metabolite relative to other endogenous estradiol metabolites has been devised and has been shown to be effective in sharply diminishing the incidence of spontaneous mammary tumors in the mouse model (3). Despite the large amount of circumstantial evidence, supporting a role for 16α-OHE1 in breast cancer initiation, the concept remains hypothetical and it is the purpose of the studies described here to obtain definitive evidence for or against the postulated scheme.

B. Background

The role of endogenous estrogens as a predisposing factor for breast cancer has been one of the central themes in the studies devoted to the biology and epidemiology of this prevalent disease. Numerous studies have been devoted to the measurement of endogenous

estrogens in the urine and plasma of breast cancer patients and normal controls in an attempt to detect differences between the two populations but no consistent and reproducible distinctions have been generally recognized (4-6). This could be due to the fact that differences in hormonal levels present at the time of the initiation of the disease would most likely be extinguished at the time of the clinical diagnosis which often occurs at a different reproductive stage and hormonal milieu of the woman.

We, therefore, pursued another strategy in which we sought to identify hormonal parameters which did not change with age or reproductive status, and which could be examined in a population of post and perimenopausal women with and without breast cancer with some assurance that any differences found would have been present at the time of disease initiation, when the women were younger and premenopausal. Specifically we established that the three main components of the oxidative metabolism of estradiol (E_2), as measured in vivo by a radiometric technique, were invariant with age (7). When measured in postmenopausal women with breast cancer, a significant increase in 16α-hydroxylation relative to a control group was found, without any differences being observed in the other two main oxidative pathways, 2-hydroxylation and 17-ol oxidation (8). Subsequent studies carried out by us and by other groups, both in humans and in animals, established a consistent pattern of increased 16α-hydroxylation of estrogens being associated with an increased risk of mammary tumor formation (9). Furthermore, studies on enzymatic activity in specific tissues indicated that in the mouse and in the human, the increased 16α-hydroxylase activity was detectable in breast tissue and more specifically in the terminal duct lobular units, the site of ductal carcinoma which represents about 80% of all human breast cancer. Estrone (E_1) functions as a common precursor that, via a competing but mutually exclusive metabolic pathway, is converted to 2-hydroxyestrone (2-OHE_1) or to 16α-

hydroxyestrone (16α-OHE_1). It is therefore important to note that because of the competing nature of 2- and 16α-hydroxylations, a decrease in the former is tantamount to an increase in the formation of the products of the latter, while conversely an increase in 2-hydroxylation results in a decrease of 16α-hydroxylated estrogens. Among a number of mammalian species studied, only the human and the mouse exhibited high 16α-hydroxylase activity consistent with their high rate of spontaneous mammary tumor formation (2, 4, 9). The activity of the 16α-hydroxylation was also examined in a series of mouse strains with differing rates of mammary tumor formation. An excellent direct correlation between 16α-hydroxylation and tumor incidence was observed (10).

C. Purpose of the Present Work

The purpose of this proposed work is to obtain direct evidence of the impact of 16α-hydroxylation in the initiation of breast cancer at the molecular and physiological level, and to identify methods for the prevention of this disease.

D. Methods of Approach

The overall approach for the experiments conducted on the cell culture and explant culture models involves i) detection of the extent of E_2 metabolism using radiometric, gas chromatographic-mass spectrometric (GC-MS) and immunoenzymatic assays (7, 8, 26), ii) evaluation of E_2 responsiveness by assays for growth regulation and for hormone receptor content, and iii) assessment of genotoxicity by unscheduled DNA synthesis assay. All of these assays have been optimized for the <u>in vitro</u> models (11, 12, 14) and are routinely being used in our laboratory.

II. Progress Report (September 1994 - August 1995)

A. Experiments Using Mouse and Human Mammary Cell Culture Systems

The primary focus of the experiments utilizing the in vitro models was to examine the role of estrogens in mammary tumorigenesis in an effort to identify mechanisms for efficacious prevention. The research during the first year funding utilized the developed murine and human mammary epithelial cell culture system as a model to examine i.) the relationship between oncogene-mediated upregulation of growth and cellular metabolism of 17ß-estradiol (E_2) and ii.) the relationship between elevated C2-hydroxylation of E_2 and inhibition of aberrant hyperproliferation. In addition, studies utilizing the mammary explant culture system have been systematically analyzed to examine whether this system represents a valid in vitro model for identifying a role for estrogens in human mammary carcinogenesis and in chemoprevention.

The following is a brief summary of experiments completed and results obtained during the first year funding of the grant #DAMD 17-94-J-4441.

1. Alteration in E_2 metabolism in mouse mammary epithelial cells expressing c-myc oncogene [collaborating investigators: Inoue, Bradlow, Telang]:

Our previous studies on immortalized, non tumorigenic murine mammary epithelial cell lines have demonstrated that exposure to chemical carcinogens or transfection with oncogenes results in induction of aberrant hyperproliferation as evidenced by anchorage-independent growth (AIG) in vitro prior to tumorigenesis in vivo as evidenced by mammary fat pad or sub-cutaneous transplantation (11-15). The experiment presented in AT-1 was

performed to examine whether relative extent of AIG corresponds with the degree of transformation in the parental MMEC, oncogene-initiated MMEC/myc_3 and oncogene-transformed MMEC/myc_3-Pr_1 cells. The AIG assay revealed a progressive increase in anchorage-independent colonies formed by MMEC/myc_3 and MMEC/myc_3-Pr_1 cells and a positive correlation with tumorigenicity in vivo. The parental MMEC cells, however, were negative in the AIG assay as well as in the tumorigenicity assay. These results suggest that AIG may represent a valid cellular marker for preneoplastic and neoplastic transformation of mammary epithelial cells.

The experiment presented in AT-2 was conducted to examine whether c-myc oncogene-induced tumorigenic transformation influences the cellular metabolism of E_2 and steroid receptor status. The relative extent of E_2 metabolism was determined by the radiometric assay measuring C2- and C16α-hydroxylation of E_2. The data is expressed as the ratio of C2/C16α-hydroxylation. The relative extent of estrogen receptor protein (ERP) and progesterone receptor protein (PRP) was determined using a commercially available immuno-assay. The C2/C16α ratio exhibited a progressive decrease in MMEC/myc_3 and MMEC/myc_3-Pr_1 cells relative to that observed in MMEC cells. The observed decrease in the ratio was due to an increase in the C16α-hydroxylation and a concomitant decrease in the C2-hydroxylation pathway of E_2 metabolism (data not shown). Consistent with the alteration in cellular metabolism of E_2, the ERP levels also decreased in MMEC/myc_3 and MMEC/myc_3-Pr_1 cells relative to those in MMEC cells. In contrast, PRP levels were found to increase in MMEC/myc_3 cells but not in MMEC/myc_3-

Pr_1 cells. These results suggest that overexpression of c-myc may induce aberrant hyperproliferation in part by influencing E_2 metabolism to increase 16α-hydroxylation and by downregulating hormone responsiveness in mammary epithelial cells.

2. Estrogen responsiveness of myc-oncogene-transformed mammary epithelial cells [collaborating investigators: Katdare, Sepkovic, Bradlow, Telang]:

The conventional determinants for estrogen response include: E_2-mediated initiation of replicative DNA synthesis, expression of early response genes, estrogen and progesterone receptor upregulation, polypeptide growth factor expression and reversible growth inhibition by antiestrogens (16, 17). In addition, oxygenated metabolites of E_2 generated via oxido-reductases or P450-dependent hydroxylases are noted to exert direct effects on hormone-responsive target tissue (18, 19). Our earlier observation that constitutive overexpression of c-myc confers aberrant hyperproliferation and partial abrogation of serum-derived growth factor requirement (15) prompted us to examine the extent of E_2 metabolism and degree of E_2 responsiveness in myc oncogene-transformed MMEC/myc_3-Pr_1 cells.

In the experiment presented in AT-3 MMEC/myc_3-Pr_1 were incubated with 10nM E_2 for 96 hours and the culture medium was used for product isolation and identification by GC/MS. The results of this experiment clearly demonstrate that the cells were capable of converting E_2 into such oxygenated metabolites as E_1, 16α-OHE_1, 2-OHE_1 and E_3. These results, together with those obtained using the radiometric assay provide a strong support to the

concept that mammary epithelial cells in culture retain the ability to metabolize E_2.

The persistence of E_2 responsiveness in MMEC/myc_3-Pr_1 cells was examined by determining whether E_2 can upregulate growth and increase ERP content in MMEC/myc_3-Pr_1 cells. The data presented in AT-4 demonstrates that a continuous 5 day exposure to E_2 resulted in a dose-dependent increase in cell number as well as in ERP levels and that at physiological levels (1-10nM), E_2 induced a substantial increase in growth and in ER and PR positivity.

3. **Modulation of E_2 metabolism and aberrant hyperproliferation in human mammary carcinoma MCF-7 cells [collaborating investigators: Suto, Zvanovec, Telang]:**

Our earlier studies on the murine mammary epithelial cell culture systems have shown that exposure to prototypic initiators of tumorigenesis (chemical carcinogens and oncogenes) results in decreased ratio of C2/C16α-hydroxylation of E_2 and increased aberrant hyperproliferation (11-14). These biochemical and cellular perturbations are reversed by the presence of known inhibitors of mammary carcinogenesis (12, 20). The experiment presented in AT-5 was designed to validate E_2 metabolism and aberrant hyperproliferation as markers for human mammary carcinogenesis. The extent of alteration in 2-OHE_1 formation as an endpoint for E_2 metabolism, and in AIG as an endpoint for aberrant hyperproliferation was measured in MCF-7 cells treated with the tumor inhibitors indole-3-carbinol (I3C), tamoxifen (TAM) and its

metabolite 4-hydroxy tamoxifen (4-OHTAM). The results presented in AT-5 demonstrate that treatment with I3C and 4-OHTAM resulted in increased 2-OHE_1 formation and decreased AIG. The inhibitory effect of TAM on AIG, however, was not accompanied by enhanced 2-OHE_1 formation, suggesting a distinct mechanism for antiproliferative effect of TAM. Treatment of MCF-7 cells with the E_2 metabolites revealed enhancement of AIG with 16α-OHE_1 but not with 2-OHE_1 treatment. Taken together, the experiment on MCF-7 cell culture system indicates that E_2 metabolism and AIG provide useful in vitro markers for inhibitors of human mammary carcinogenesis and that specific metabolites of E_2 exert distinct modulatory influence on the growth of MCF-7 cells in culture.

4. **Application of mammary explant culture system and mammary epithelial cell culture system as models for identifying the role of estrogens in mammary carcinogenesis [collaborating investigators: Telang, Fishman, Osborne]:**

In vitro models derived from mammary explant and cell culture systems provide an innovative approach to examine the effects of exogenous agents on the process of mammary carcinogenesis and its modulation directly at the level of target tissue (11, 13, 20-24). Earlier experiments conducted on murine mammary explant culture system have shown that treatment of the non-involved mammary tissue to chemical carcinogens results in increased carcinogen-DNA binding, enhanced C16α-hydroxylation of E_2 and high incidence of aberrant hyperplasia in vitro, prior to tumorigenicity in vivo (21-25). Independent studies utilizing the epithelial cell culture model have also

shown that treatment with carcinogens or transfection with oncogenes results in upregulation of same biochemical and cellular endpoints (11, 13-25). The results obtained from the two in vitro models therefore provide strong evidence that perturbed biochemical and cellular events detectable prior to the appearance of cancer represent markers for preneoplastic transformation.

The experiment presented in AT-6 was designed to examine the relationship between the extent of E_2 metabolism via C16α-hydroxylation and breast cancer risk in murine and human mammary explant cultures. The relative extent of E_2 C16α-hydroxylation was found to increase about 3-5 fold in the explants from the high risk groups relative to the respective low risk groups. In addition, explants from the high risk groups exhibited a greater response to carcinogen-induced perturbation of E_2 C16α-hydroxylation (data not shown). The data generated with the experiments on the mammary explant culture model indicates that E_2 C16α-hydroxylation represents a biochemical marker whose alteration may predict the susceptibility of the target tissue to carcinogenesis.

The experiment to examine whether E_2 metabolites generated via the C16α-hydroxylation pathway may function as initiators of mammary carcinogenesis utilized the mouse mammary epithelial cells as the model (AT-7). Unscheduled DNA synthesis (UDS) and anchorage-independent growth (AIG) represented the biomarkers for genotoxicity and aberrant hyperproliferation respectively. From amongst the metabolites tested, only 16α-hydroxyestrone (16α-OHE_1) exhibited maximal induction in UDS and AIG. The upregulation of the two endpoints was qualitatively similar to that

observed in cells treated with genotoxic chemical carcinogen (positive control).

B. Molecular Aspects of 16α-hydroxylase

To examine the molecular aspects of 16α-hydroxylase, two experimental approaches have been utilized during the first year of funding. First, we have initiated studies to test the hypothesis that 16α-hydroxyestrone has unique effects on estrogen receptor function. Second, we have made substantial progress in isolating cDNA clones which encode estrogen 16α-hydroxylase activity. Conclusive identification of the gene(s) encoding this activity will allow us to define the mechanisms by which 16α-hydroxylated estrogens are overproduced and open new avenues for diagnosis and prevention of breast cancer. The data obtained in the initial year of funding are briefly summarized below.

1. Effects of 16α-hydroxyestrone on estrogen receptor function

In order to perform informative studies relating to this issue, we first needed to establish two important criteria in our experimental system. First, we wished to establish that the MCF-7 cells were inducing gene expression in an estrogen-dependent manner. This is especially important because of the reported heterogeneity of MCF-7 cells in different laboratories. Second, we wanted to obtained evidence that estrogen receptor-dependent gene expression could be selectively altered by 16α-hydroxyestrone in these cells. We used pS2 expression as our marker for these studies, because it is transcriptionally regulated by estrogens in MCF-7 cells. pS2 mRNA is virtually undetectable in MCF-7 cells incubated in media without estrogens,

but is induced 20- to 40-fold after exposure to estradiol (27). We found that most of the lots of MCF-7 cells made available to us were suboptimal because they contained significant basal levels of pS2 mRNA which were poorly induced by estrogens. However, one lot (a gift from Ann Bowcock, University of Texas Southwestern Medical Center, Dallas, TX) exhibited barely detectable levels of pS2 mRNA which was strongly induced by estradiol. These cells have been chosen for use in our subsequent studies.

To obtain functional evidence that ER function might be selectively altered by exposure to 16α-hydroxyestrone, we stimulated MCF-7 cells with estradiol, 16α-hydroxyestrone, or estriol for 1 or 14 days. We then removed the hormone for 5 days and measured pS2 promoter function by transfection with a pS2-CAT reporter plasmid (AT-8). Cells pretreated with estradiol or estriol for either length of time displayed virtually no CAT activity, except when re-exposed to estradiol post-transfection, where a marked induction was observed. On the other hand, cells pretreated with 16α-hydroxyestrone for 14 days had substantial CAT activity which was only marginally induced by re-exposure to estradiol. Indeed, the reporter activity in the 16α-hydroxyestrone-pretreated cells was about 20% of the levels observed in "virgin" cells treated with estradiol for 48 hr post-transfection. None of these effects were observed in cells which had been pretreated with 16α-hydroxyestrone for 1 day. These data support the concept that an "irreversible" interaction between ER and 16α-hydroxyestrone can cause a sustained response in estrogen-responsive genes. We have isolated nuclear extracts from MCF-7 cells stimulated by the above paradigm, which we are using to evaluate directly the effect of these

estrogens on ER binding to its cognate element.

2. Isolation of cDNA clones with estrogen 16α-hydroxylase activity

In order to clone the estrogen 16-hydroxylase, we have taken advantage of murine T_1Pr_1 and human MCF-10 cell lines, which contain exceptionally high levels of this activity (28, 29). Inhibition studies strongly suggest that the 16α-hydroxylase is a member of the cytochrome P450 superfamily of mono-oxygenases. Hence, our strategy has relied on PCR amplification with degenerate primers that correspond to conserved regions in certain P450 families. We have previously used a similar PCR-based approach to clone rare homeodomain-containing proteins (30). Additionally, others have recently used this strategy to identify a novel DMBA-inducible P450 species in fibroblasts (31). We have designed three sets of degenerate primers, which should amplify P450 sequences from families 1, 2, or 3. We suspect that the unidentified estrogen 16α-hydroxylase may fall within these classes for the following reasons. First, CYP1A1 is the best candidate for the breast-specific estrogen 2-hydroxylase (32) and CYP1B1 may be responsible for the estrogen 4-hydroxylase activity observed in MCF-7 cells. Hence, it is reasonable to postulate that the CYP gene encoding estrogen 16α-hydroxylase activity might also reside within the CYP1 family. The rationale for considering the CYP2 family is based on the observation that the mouse male-specific testosterone 16α-hydroxylase belongs to the *Cyp2d* subfamily and the rat female-specific 15ß-hydroxylase lies within the CYP2C cluster (33). Hence, CYP family 2 contains sexually dimorphic genes which encode activities

closely related to estrogen 16α-hydroxylase. Finally, we have considered family 3 because certain members, such as CYP3A4, exhibit a remarkably diverse array of activities which includes the metabolism of endogenous steroids.

Thus far, two sets of degenerate primers (specific for families 1 or 2) have been used successfully to amplify fragments of the correct size, using either T_1Pr_1 or MCF-10 cDNA as template. Because these fragments may be heterogeneous, we have subcloned them into the vector pGEM-T (Promega) and numerous clones have been analyzed by DNA sequencing. For family 1, all clones analyzed thus far correspond to CYP1A2, which is a stronger estrogen 2-hydroxylase than CYP1A1. This is somewhat surprising, because previous reports had indicated that CYP1A1 and CYP1B1, but not CYP1A2, are present in human MCF-7 cells. Our primers amplify CYP1A1, CYP1A2 and CYP1B1 with similar efficiency, so we entertained the hypothesis that CYP1A2 may indeed be preferentially expressed in these cells. Indeed, Northern analysis indicates that CYP1A2 levels are significantly higher than CYP1A1 in $T1Pr_1$ cells. We do not know the basis for this heterogeneity of expression for CYP1A members in mammary cell lines at present, but our observations emphasize the need to evaluate CYP1A gene expression in vivo very carefully. This has yet to be done in the breast. In any event, since CYP1A2 does not 16-hydroxylate estrogens, and no other CYP1 PCR fragments have been detected in our analysis to date, we have adopted the working hypothesis that the 16-hydroxylase is not a CYP1 family member.

Using the family 2 primers and T_1Pr_1 cDNA as template, we have amplified a particularly strong DNA fragment of the correct size. We have sequenced 16 clones derived from this fragment, all of which correspond to a novel mouse *Cyp2-d* family member. We have yet to obtain a full-length clone corresponding to this PCR fragment (currently termed *Cyp2-dx*), but several observations suggest that *Cyp2-dx* may correspond to a bona fide estrogen 16α-hydroxylase. First, the clone is similar to a rat *Cyp2-d* member termed CMF-3 (34) (Fig. 1). CMF-3 was isolated by screening a rat liver cDNA library with a monclonal antibody raised against a purified P450 with estrogen 16α-hydroxylase activity. Second, *Cyp2-dx* RNA levels are very high in T_1Pr_1 and RIII/Pr1 cells, but barely detectable in MMEC and RIII/MG cells. This correlates with the observed 16α-hydroxylase activities for these cells. Third, *Cyp2-dx* is also highly related to murine *Cyp2d-9*, which encodes testosterone 16α-hydroxylase activity (Fig. 1).

Once a full-length clone has been obtained for *Cyp2-dx*, we will need to examine its ability to 16α-hydroxylate estrogens. This is best accomplished by stable expression in a mammalian cell line with low endogenous 16α-hydroxylase activity. MCF-7 cells are an appropriate choice for our purposes; they 16-hydroxylate poorly, are easily transfectable and we have found that they contain undetectable levels of *Cyp2-d*-related RNA species. Moreover, it would be of additional benefit if the test gene were inducible, so that its expression would be minimal during generation of the stable line. We have evaluated a tetracycline-dependent expression system in MCF-7 cells, and found it to work extremely well. This system requires two components; 1) a plasmid that encodes a tet repressor/VP16 fusion protein, termed TTA, and 2) a *tet* operator-controlled expression plasmid that drives the expression of the gene of interest (35). TTA is a potent repressor in the presence of tetracycline, but permits potent activation of the *tet*-controlled gene in the absence of

tetracycline. Using a firefly luciferase cDNA to test this system in MCF-7 cells, we have observed that removal of tetracycline from the culture medium induces luciferase activity by more than 200-fold. Tetracycline does not influence *Cyp2-dx* levels in T_1Pr_1 cells; hence, this should be an excellent system to evaluate the capacity of *Cyp2-dx* to 16α-hydroxylate estrogens.

III. General Conclusions

In the first year of the grant the foundation has been constructed from which a clarification of the mechanism of 16α-OHE_1 action in breast cancer can be achieved. We have carried out experiments in cell culture systems demonstrating the unique ability of 16α-OHE_1 to bring about hyperproliferation with increased anchorage-independent growth. This, as well as other studies has indicated the carcinogen like activity of 16α-OHE_1. We have also carried out studies with mouse and human explant cultures of two types (high and low risk for breast cancer) and a marked increase in 16α-hydroxylation was observed with cultures from the high risk mice or women. The differences demonstrated in these non-cancerous tissues indicate the importance of a chronic increase in estrogen 16α-hydroxylation prior to the development of breast tumors.

The experiments to identify the cytochrome P450 gene which catalyzes estrogen 16α-hydroxylation have indicated that it is probably *Cyp2-dx*. Further studies should confirm whether or not it is this gene which is responsible for the enzyme. Once identified it may be possible to alter the expression of this gene in breast cells and therefore reduce the risk of breast cancer.

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AT-1

Induction of Aberrant Hyperproliferation and Tumorigenic Transformation by C-myc Oncogene in Mouse Mammary Epithelial Cells.

Cell Line	Status of Transformation	
	in vitro	**in vivo**
	Anchorage-independent growth (%CFE)[a]	**Tumorigenecity[b]**
MMEC	ND[c]	ND[c]
MMEC/myc_3	12.8 ± 3.8	7/10
MMEC/myc_3-Pr_1	26.8 ± 3.8	10/10

[a] $\frac{\text{Number of colonies}}{\text{Initial seeding density}} \times 100$

[b] $\frac{\text{Number of tumors}}{\text{Number of transplant sites}}$

[c] ND: not detected

AT-2

C-myc Oncogene-Mediated Alteration in Estradiol Metabolism and in Hormone Receptor Status

Cell Line	E_2 Metabolism[a] (C2/C16α-hydroxylation)	Receptor Protein Content[b] (fmoles per 1.0 x 10^6 cells)	
		ERP	PRP
MMEC	5.4 ± 0.4	11.9 ± 0.5	0.8 ± 0.3
MMEC/myc_3	2.7 ± 0.4	8.8 ± 1.5	2.4 ± 0.4
MMEC/myc_3-Pr_1	1.6 ± 0.6	2.9 ± 0.5	1.6 ± 0.1

[a] determined by $^3H_20$ formation after incubation with [C2-^{3}H]E_2 and [C16α-^{3}H]E_2

[b] determined by immunoassay

AT-3

Metabolism of 17ß-Estradiol (E_2) in MMEC/myc_3-Pr_1 Cells

E_2 Metabolites	Relative Abundance [a,b] (Normalized per 1.0 x 10^7 cells)
E_1	1465 ± 304
16α-OHE_1	206 ± 20
2-OHE_1	799 ± 178
E_3	190 ± 14

[a] determined by the RP-HPLC and GC-MS analysis of the culture medium

[b] MMEC/myc_3-Pr_1 cells incubated with 1.0 x 10^{-8}M E_2 for 96 hours

AT-4

Ligand-mediated Upregulation of Proliferation and Hormone Receptor Status in MMEC/myc_3-Pr_1 Cells

E2 Treatment (nM)	Increase in cell number[a] ($x10^5$)	Receptor Protein Content[b] (fmoles/1.0 x 10^6 cells)	
		ERP	PRP
0.0	2.3 ± 0.6	2.1 ± 0.4	1.3 ± 0.2
0.1	2.8 ± 0.4	2.9 ± 0.5	1.6 ± 0.2
1.0	4.2 ± 0.5	8.0 ± 1.0	2.9 ± 0.2
10.0	4.4 ± 0.5	13.7 ± 3.1	3.5 ± 0.3

[a] cells maintained in serum-free medium for 5 days

[b] determined by immunoassay

AT-5

Modulation of Estradiol 2-Hydroxylation and Aberrant Hyperproliferation in Human Mammary Carcinoma MCF-7 Cells

Agent	2-hydroxylation of E_2[a] (% per 1.0 x 10^4 cells)	Anchorage-Independent Growth (% CFE)[b]
EtOH		
(solvent control)	2.4 ± 0.4	15.3 ± 1.4
I3C	6.2 ± 0.3	5.0 ± 1.1
TAM	1.6 ± 0.1	3.7 ± 0.7
4-OH TAM	3.6 ± 0.2	1.8 ± 0.2
2-OHE_1	ND[c]	4.9 ± 2.9
16α-OHE_1	ND[c]	25.9 ± 2.0

[a] determined from $^3H_20$ formation after a 48 hour incubation with [C2-^{3}H] E_2

[b] $\frac{\text{Number of colonies}}{\text{Initial seeding density}}$ x 100

[c] ND: not done

AT-6

Breast Cancer Risk-Dependent Enhancement of Estradiol 16α-Hydroxylation in Murine and Human Mammary Explant Cultures

Origin	Cancer Risk	Tissue Type[a]	Estradiol C16α-hydroxylation (% per mg tissue)[b]
Murine	Low	NFS-MDE	0.16 ± 0.05
	High	C3H-MDE	0.59 ± 0.13
Human	Low	TDLU-LR	0.05 ± 0.02
	High	TDLU-HR	0.31 ± 0.05

[a] Mammary ductal epithelium from low risk NFS strain, mammary ductal epithelium from high risk C3H strain, terminal duct lobular units from reduction mammoplasty, terminal duct lobular units from cancer mastectomy

[b] determined from $^3H_20$ formation after a 48 hour incubation with [C16α-^{3}H] E_2]

AT-7

Induction of Unscheduled DNA Synthesis and Aberrant Hyperproliferation by Estradiol Metabolites in Mouse Mammary Epithelial C57/MG Cells

Agent	Unscheduled DNA Synthesis (HU-insensitive ^{3}H-thymidine uptake, cpm x 10^6/mg DNA)	Anchorage-Independent Growth (% CFE)[a]
DMSO	12.5 ± 1.0	ND[b]
(solvent control)		
DMBA	21.4 ± 2.2	24.0 ± 2.4
(positive control)		
E_2	12.5 ± 1.0	1.0 ± 0.5
16α-OHE_1	22.0 ± 3.5	5.5 ± 0.3
2-OHE_1	13.0 ± 0.9	0.3 ± 0.2

[a] $\frac{\text{Number of colonies}}{\text{initial seeding density}} \times 100$

[b] ND: not detected

pS2-CAT activity in MCF-7 cells pre-exposed to estrogens

Pretransfection Hormone	Duration	Postransfection Treatment	CAT Activity
Ethanol	1	Ethanol	0.5
	1	E_2	19.7
Ethanol	14	Ethanol	0.3
	14	E_2	17.4
16αOHE$_1$	1	Ethanol	0.5
	1	E_2	20.2
16αOHE$_1$	14	Ethanol	**5.7**
	14	E_2	18.9
Estradiol	1	Ethanol	0.6
	1	E_2	22.4
Estradiol	14	Ethanol	0.5
	14	E_2	16.9
Estriol	1	Ethanol	0.4
	1	E_2	17.8
Estriol	14	Ethanol	0.4
	14	E_2	20.0

MCF-7 cells were treated for 1 or 14 days with the indicated hormone at a concentration of 10^{-8}M. Cells were then incubated in the absence of hormone for 5 days. After transfection with a pS2-CAT reporter, cells were incubated in the presence or absence of 10^{-8}M estradiol for 48 hours and harvested for CAT asssay. CAT activity indicates % conversion of chloramphenicol to monoacetylated forms.

Fig. 1

Alignment of *Cyp2-dx* with rat CYP2D4 (P450-CMF3) and mouse *Cyp2-d9* (testosterone 16α-hydroxylase)

```
                10         20         30         40         50         60

Cyp2-dx   TGCGGTGGTG CATGAGGTGC AGCGCTTTGC AGACATTCTC CCTCTTGGTG TACCTCACAA

CYP2D4    TGCtGTcaTc CATGAGGTGC AGCGCTTTGC AGACATTCTC CCTCTTGGTG TgCCTCACAA
          ^^^v^^vv^v ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^v^^^^^^^^

Cyp2-d9   TGCtGTcaTt CATGAGGTGC AGCGCTTTGg gGACATTgTt CCagTgaaTt TgCCaCgCAt
          ^^^v^^vv^v ^^^^^^^^^^ ^^^^^^^^^v v^^^^^^v^v ^^vv^vvv^v ^v^^v^v^^v


                70         80         90        100        110        120

Cyp2-dx   GACTTCTCGT GACATTGAAC TACAGGGCTT CCTTATCCCT AAGGGACGAC CCTCATCACC

CYP2D4    GACTTCTCGT GACATTGAAg TgCAGGGCTT CCTTATCCCT AAGGGACaAC CCTCATCACC
          ^^^^^^^^^^ ^^^^^^^^^v ^v^^^^^^^^ ^^^^^^^^^^ ^^^^^^^v^^ ^^^^^^^^^^

Cyp2-d9   cACaagTCaT GACATTGAAg TgCAGGaCTT CCTcATCCCc AAGGGACGAt CCTCcTCcCC
          v^^vvv^^v^ ^^^^^^^^^v ^v^^^^v^^^ ^^^v^^^^^v ^^^^^^^^^v ^^^^v^^v^^


                130        140        150        160

Cyp2-dx   AACCTGTCCT CCGCGCTAAA AGATGAGACT GTCTGGGAGA AGCCCTCT

CYP2D4    AACCTGTCCT CaGtGCTgAA gGATGAGACc GTCTGGGAGA AGCCC-CT
          ^^^^^^^^^^ ^v^v^^^v^^ v^^^^^^^^v ^^^^^^^^^^ ^^^^^ ^^

Cyp2d-9   AACaTGTCCT CCatGCTgAA AGATGAGtCT GTCTGGGAGA AGCCC-CT
          ^^^v^^^^^^ ^^vv^^^v^^ ^^^^^^^v^^ ^^^^^^^^^^ ^^^^^ ^^
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The Role of Estrogen in Mammary Carcinogenesis

JACK FISHMAN AND NITIN T. TELANG
Strang-Cornell Cancer Research Laboratory
Cornell University Medical College
New York, New York 10021

INTRODUCTION

Human breast cancer is one of the most prevalent diseases in women. The American Cancer Society estimates approximately 182,000 new cases of breast cancer and about 46,000 breast cancer–related deaths for 1994.[1] The epidemiological evidence provides strong support for the concept that environmental, hormonal, and genetic factors affect the risk and development of breast cancer.[2–6] From among the various recognized risk factors, age at menarche, age at first pregnancy, and age at menopause suggest that endogenous ovarian steroids may profoundly affect initiation, promotion, and progression of carcinogenesis.[3,6,7]

Experimental studies on rodent mammary tissue have demonstrated that the ovarian steroids, 17β-estradiol (E_2) and progesterone (Prg), acting in concert, induce proliferation and positively regulate mammary epithelial morphogenesis, that is, formation of the epithelial ductal system exhibiting proliferative terminal end-buds (TEB). In response to such mammotropic/lactogenic hormones as adrenal glucocorticoids and the pituitary polypeptide prolactin, TEB are induced for functional cytodifferentiation as evidenced by the presence of transformation to secretory lobulo alveoli. The selected steroid and polypeptide hormones that are mammotropic in the rodent mammary system are also reported to regulate epithelial cell proliferation in human mammary terminal duct lobular units (TDLU), the principal target site for carcinogenesis in humans.[5,7,8]

From among the mammotropic hormones as endogenous factors that influence mammary carcinogenesis, estrogens have attracted the most attention. The natural estrogen E_2 is a well-known promoter of rodent mammary carcinogenesis. Carcinogen-induced as well as spontaneous mammary tumors in rodent models are negatively growth-regulated by surgical or chemical ablation of ovarian function.[2,3,5–7,9,10] E_2 induces DNA synthesis in quiescent cells, increases the expression of oncogenes, and functions as a potent mitogen in estrogen-responsive tissues.[3,6,11] Taken together, these observations provide evidence that estrogens, because of their mitogenic property, may increase the susceptibility of the target tissue to initiation. Additionally, E_2-mediated mitogenicity on preinitiated target cells may predispose these cells to tumorigenic transformation.

Microsomal hydroxysteroid dehydrogenases and P450-dependent steroid hydroxylases are critical for the estrogen biotransformation that converts highly estrogenic E_2 to progressively less estrogenic metabolites.[12,13]

In vivo experiments utilizing strains of mice that differ in their relative risk of developing the murine mammary tumor virus (MTV)–induced mammary adenocarci-

Estrogen Metabolite Ratios and Risk Assessment of Hormone-related Cancers

Assay Validation and Prediction of Cervical Cancer Risk

D. W. SEPKOVIC,[a] H. L. BRADLOW,[a] G. HO[b]
S. E. HANKINSON,[c] L. GONG,[a] M. P. OSBORNE,[a]
AND J. FISHMAN[a]

[a]*Strang-Cornell Cancer Research Laboratories*
New York, New York 10021
[b]*Department of Epidemiology and Social Medicine*
Albert Einstein College of Medicine
Bronx, New York 10461
[c]*Channing Laboratory*
Brigham and Women's Hospital
and
Harvard Medical School
Boston, Massachusetts 02115

INTRODUCTION

Estrogens are believed to act as initiation promoters in breast malignancies causing increased growth rates in transformed cells. The major metabolites of estradiol are 16α-hydroxylated at either the C-2 or C-16α positions, with a much smaller amount at the C-4 position. C-2 metabolites represent the irreversible biotransformation of estradiol to compounds that are essentially devoid of potent estrogenic activity.[1] C-2 and C-16α hydroxylation are competitive alternatives and increases in the activity of one of these two oxidative pathways will result in a diminution of metabolites formed via the alternative pathway.[2] The principal C-2 metabolite in humans is 2-hydroxyestrone (2-OHE_1). Recent studies have shown that 2-OHE_1 is weakly antiestrogenic.[3]

The other major pathway of estradiol metabolism, C-16α hydroxylation, results in the formation of 16α-hydroxyestrone (16α-OHE_1) and estriol (E_3). Both of these compounds retain significant estrogen agonist activity. 16α-OHE_1 is a uniquely reactive estrogen that is known to be present in humans at levels that are comparable to free estradiol.[4] This estradiol metabolite is capable of covalently binding to estrogen receptors, to nuclear histone proteins, and to other amino-containing compounds.[5–7] The formation of 16α-OHE_1 is elevated in women with breast cancer, in women at high risk for breast cancer, and in strains of mice with a high incidence of spontaneous mammary tumors.[8–10]

In the present study, we utilize an enzyme-linked immunoassay for these metabolites in urine[11] and correlate this endocrine biomarker with cervical intraepithelial neoplasia

Effects of Pesticides on the Ratio of 16α/2-Hydroxyestrone: A Biologic Marker of Breast Cancer Risk

H. Leon Bradlow,[1] Devra Lee Davis,[2] Gong Lin,[1] Daniel Sepkovic,[1] and Raj Tiwari[1]

[1]Strang-Cornell Cancer Research Laboratory, New York, New York; [2]Office of the Assistant Secretary for Health, Dept. of Health and Human Services, Washington, DC

Xenobiotic estrogens are external compounds with estrogenic activity that may thereby affect the risk of breast cancer. This paper describes a mechanism by which xenoestrogens may affect the development of breast cancer. Estradiol metabolism proceeds by hydroxylation at one of two mutually exclusive sites at C-2 and C-16α. The catechol pathway yields the weakly estrogenic 2-hydroxyestrone (2-OHE_1), which inhibits breast cell proliferation. In contrast, the alternative pathway yields the genotoxic 16α-hydroxyestrone (16α-OHE_1), which enhances breast cell growth, increases unscheduled DNA synthesis, oncogene and virus expression, and increases anchorage-independent growth. Using a radiometric assay that measures the relative formation of 16α-OHE_1 versus 2-OHE_1 from specifically tritiated estradiol in (ER+) MCF-7 cells, we compared the ratio of 16α-OHE_1/2-OHE_1 observed after treatment with the known rodent carcinogen DMBA with the ratios after treatment with DDT, Atrazine, γ-Benzene hexachloride, Kepone, coplanar PCBs, Endosulfans I and II, linoleic and eicosapentenoic acids, and indole-3-carbinol (I3C). These pesticides significantly increase the ratio of 16α-OHE_1/2-OHE_1 metabolites to values comparable or greater than those observed after DMBA. In contrast, the antitumor agent I3C increased 2-OHE_1 formation and yielded ratios that are 1/3 of those found in unexposed control cells and 1/10th of those found in DMBA-treated cells. Thus the ratio of 16α-OHE_1/2-OHE_1 may provide a marker for the risk of breast cancer. Assays of this ratio, which can be measured in spot urines, may prove useful for a variety of *in vitro* and *in vivo* studies bearing on breast cancer risk. — Environ Health Perspect 103(Suppl 8):00–00 (1995)

Key words: Estrogens, P450-hydroxylation, pesticides, cancer risk, hydroxyestrogens

Introduction

Changes in screening practices or in known or suspected risk factors cannot completely account for recently observed increases in the incidence of breast, prostate, and testicular cancer or suspected increases in male and female reproductive disorders, such as reduced sperm count, increased reproductive failures, endometriosis, and ovarian fibroid tumors. With respect to breast cancer, most of the known risk factors other than genetic makeup for the disease can be related to cumulative lifetime exposure to estrogen (*1,2*).

We have recently hypothesized that foreign compounds with estrogenic activity can affect the risk of breast cancer (*3*). Estradiol metabolism predominantly proceeds via two mutually exclusive pathways: one pathway yields the catechol estrogen 2-hydroxyestrone (2-OHE_1), which is weakly antiestrogenic and nongenotoxic (*4*); the alternative pathway yields 16α-hydroxyestrone (16α-OHE_1), a fully potent estrogen, which is tumorigenic and genotoxic and causes increased cell proliferation (Figure 1) (*5*). The reasons for suspecting that alterations in endocrine function underlie estrogen-mediated cancers, such as those of the breast and endometrium, have evolved from diverse observations in endocrinology, biochemistry, and epidemiology. Some 30 years ago, it was calculated that on a molar basis ~~thus~~ 2-OHE_1 possessed about 0.25% of the uterotrophic activity of estradiol (*6*). Indeed some studies suggest that 2-OHE_1 is a weak antiestrogen (*7,8*). Others have noted that 4-OHE_2 is carcinogenic in the Syrian hamster kidney model where it is a major metabolite (*9*), but it is a minor metabolite in people. In contrast, 16α-OHE_1 covalently binds with estrogen receptors and amino functions on DNA (*10,11*) and exerts persistant biological responses (*12*). A number of lines of evidence suggest that 16α-hydroxylation is a biological marker of risk for breast cancer and may directly contribute to the initiation and progression of the disease. 16α-OHE_1, the product of the 16α-hydroxylation pathway of E_2 metabolism, causes prolonged growth responses by virtue of its ability to bind covalently to the estrogen receptor (*10*). This paper presents results of an established assay of estradiol metabolism applied to a number of organochlorine pesticides. Materials that increase the ratio of 16α-OHE_1/2-OHE_1 should be regarded as potential breast carcinogens.

Methods

Estradiol metabolism via the C-2 and C-16α hydroxylation pathways was assayed radiometrically (*13*). We measured the amount of estradiol metabolized by these two pathways in estrogen receptor positive (ER+) human breast cell cultures (MCF-7) in the presence and absence of DMBA and linoleic acid as positive tumorigenic con-

This paper was presented at the Symposium on Estrogens in the Environment, III: Global Health Implications held 9–11 January 1994 in Washington, DC.

We are indebted to the Noreen T. Holland Foundation for generous support of this work.

Address correspondence to Dr. H.L. Bradlow, Laboratory of Biochemical Endocrinology, Strang-Cornell Cancer Research Laboratory, 510 East 73rd Street, New York, NY 10021. Telephone (212)734-0557, ext 11. Fax (212)472-9471.

Abbreviations used: 2-OHE_1, 2-hydroxyestrone; 16α-OHE_1, 16α-hydroxyestrone; ER, estrogen receptor, DMBA, 9,10-dimethylbenz(a)anthracene; E_2, estradiol; ER+, estrogen receptor positive; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; ^3H_2O, tritiated water; DDT, dichlorodiphenyltrichloroethylene.

11/94

Reliability of an Enzyme Immunoassay for Simultaneous Quantitation of 2- and 16α-hydroxyestrone in Urine of Premenopausal Healthy Women

Araxi Pasagian-Macaulay*, H. Leon Bradlow**, Daniel W. Sepkovic**, Alhaji M. Buhari*, Rena R. Wing*, Lewis H. Kuller*, Elaine N. Meilahn*

*Department of Epidemiology
Graduate School of Public Health
University of Pittsburgh
130 DeSoto Street
Pittsburgh, Pennsylvania 15261, USA

**Strang Cornell Cancer
Reasearch Laboratory
Cornell University
510 East 73rd Street
New York, New York 10021, USA

Address reprint requests to:
Dr. Araxi Pasagian-Macaulay
University of Pittsburgh
3600 Forbes Avenue, Suite 600
Pittsburgh, Pennsylvania 15213

Running Title: Reliability of measurements of 2- and 16α-hydroxylation in urine of premenopausal women.

This study was supported by the National Cancer Institute Grant # CA63401.

KEYWORDS

2-hydroxyestrone, 16α-hydroxyestrone, estrogen metabolism, reliability